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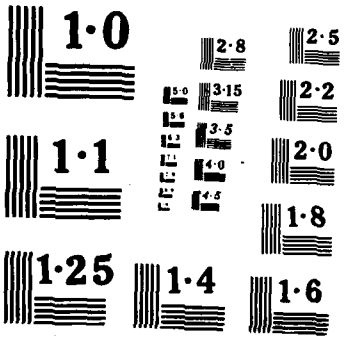
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19 ABSTRACT (Continue on reverse if necessary and identify by block number)

Four neuron-specific phosphoproteins and calcium/calmodulin-dependent protein kinase II were used as model proteins to investigate the role of protein phosphorylation in the regulation of bioreactivity in the nervous system. These studies were carried out at the levels of electrophysiology, biochemistry, and molecular biology, in an attempt to obtain the most comprehensive understanding of their functions. Synapsin I and calcium/calmodulin-dependent protein kinase II were pressure-injected into the preterminal digit of the squid giant synapse to test directly the possible regulation of neurotransmitter release by these substances. The binding of Synapsin I to small synaptic vesicles was examined. The regulation of calcium/calmodulin-dependent protein kinase II autophosphorylation and its effect on the activity of the enzyme were studied. The regional and subcellular distributions of proteins IIIa and IIIb in the nervous system were determined. The regional and subcellular distributions of protein p38 in the nervous system were determined. A partial cDNA clone for Synapsin I was obtained.

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Progress report for Air Force Molecular Biology Grant, 3/11/86

1. SUMMARY - ABSTRACT OF TECHNICAL PROGRESS

Four neuron-specific phosphoproteins and calcium/calmodulin-dependent protein kinase II were used as model proteins to investigate the role of protein phosphorylation in the regulation of bioreactivity in the nervous system. These studies were carried out at the levels of electrophysiology, biochemistry, and molecular biology, in an attempt to obtain the most comprehensive understanding of their functions. Synapsin I and calcium/calmodulin-dependent protein kinase II were pressure-injected into the preterminal digit of the squid giant synapse to test directly the possible regulation of neurotransmitter release by these substances. The binding of Synapsin I to small synaptic vesicles was examined. The mechanism of calcium/calmodulin-dependent protein kinase II autophosphorylation and its effect on the activity of the enzyme were studied. The regional and subcellular distributions of proteins IIIa and IIIb in the nervous system were determined. The regional and subcellular distributions of protein p38 in the nervous system were determined. A partial cDNA clone for Synapsin I was obtained.

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2. RESEARCH OBJECTIVES

The purpose of this research is to attempt to increase the bioreactivity of the nervous system by modifying synaptic transmission at the biochemical level. Since protein phosphorylation appears to be directly involved in synaptic transmission, this study focuses on those aspects of the protein phosphorylation system which are susceptible to external manipulations. Various conditions which alter neuronal function will be studied for their effect on the following parameters of protein phosphorylation (a) the state of phosphorylation of neuron-specific phosphoproteins (b) transcription of mRNA specific for selected protein kinases and substrate phosphoproteins and (c) synthesis of these selected protein kinases and substrate phosphoproteins by translation of the specific mRNAs.

The specific objectives are (a) to characterize the biochemical aspects of the neuron-specific phosphoproteins, (b) to isolate molecular clones for selected specific protein kinases and substrate phosphoproteins, (c) to determine the nucleotide and peptide sequences of these kinases and phosphoproteins, (d) to determine the normal time course for the expression of the mRNAs coding for these proteins (transcription) in the developing brain, (e) to determine the normal time course for the expression of these proteins (translation) in the developing brain, (f) to determine which factors affect the expression of these proteins in the normal adult brain and, (g) to determine which external factors can affect the expression of these proteins.

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3. STATUS OF THE RESEARCH

INJECTION OF SYNAPSIN I AND CALCIUM/CALMODULIN PROTEIN KINASE II

Synapsin I and calcium/calmodulin-dependent protein kinase II were pressure-injected into the preterminal digit of the squid giant synapse to test directly the possible regulation of neurotransmitter release by these substances. Neurotransmitter release was determined by measuring the amplitude, rate of rise, and latency of the postsynaptic potential generated in response to presynaptic depolarizing steps under voltage clamp conditions. Injection of dephosphosynapsin I decreased the amplitude and rate of rise of the postsynaptic potential, whereas injection of either phosphosynapsin I or heat-treated dephosphosynapsin I was without effect. Conversely, injection of calcium/calmodulin-dependent protein kinase II, which phosphorylates synapsin I on site II, increased the rate of rise and amplitude and decreased the latency of the postsynaptic potential. The effects of these proteins were observed without any detectable change in the initial phase of the presynaptic calcium current. A synapsin I-like protein and calcium/calmodulin-dependent protein kinase II were demonstrated by biochemical and immunochemical techniques to be present in squid nervous tissue. The data support the hypothesis that synapsin I regulates the availability of synaptic vesicles for release; we propose that calcium entry into the nerve terminal activates calcium/calmodulin-dependent protein kinase II, which phosphorylates synapsin I on site II, dissociating it from the vesicles and thereby removing a constraint in the release process.

BINDING OF SYNAPSIN I TO SYNAPTIC VESICLES

The binding of synapsin I, a synaptic vesicle-associated phosphoprotein, to small synaptic vesicles has been examined. For this study, synapsin I was purified under non-denaturing conditions from rat brain, using the zwitterionic detergent 3-((3-cholamidopropyl)-dimethylammonio)-1-propanesulfonate (CHAPS)[1], and characterized. Small synaptic vesicles were purified from rat neocortex by controlled pore glass chromatography as the last purification step, and binding was characterized at an ionic strength equivalent to 40 mM NaCl. After removal of endogenous synapsin I, exogenous dephospho-synapsin I bound with high affinity (Kd, 10 $\pm$ 6 nM) to synaptic vesicles. The binding saturated at 76 $\pm$ 40 μg synapsin I per mg of vesicle protein, which corresponded to the amount found endogenously in purified vesicles. Synapsin I binding exhibited a broad pH optimum around pH 7. Other basic proteins, specifically myelin basic protein and histone H2b, did not compete with synapsin I for binding to vesicles. Other membranes purified from rat brain and membranes derived from human erythrocytes did not show the high affinity binding site for synapsin I found in vesicles.

The binding of three different forms of phospho-synapsin I to vesicles was investigated. Synapsin I, phosphorylated at sites 2 and 3 by purified calcium/calmodulin-dependent protein kinase II, bound with a five-fold lower affinity to the vesicles than did dephospho-synapsin I. In contrast,

synapsin I, phosphorylated at site 1 by purified catalytic subunit of cAMP-dependent protein kinase, bound with an affinity close to that of dephospho-synapsin I. Synapsin I phosphorylated on all three sites bound to the vesicles with an affinity comparable to that of synapsin I phosphorylated on sites 2 and 3. Under conditions of higher ionic strength (150 mM NaCl equivalent), synapsin I bound with a five-fold lower affinity to vesicles, and no effect of phosphorylation on binding was observed under these conditions.

AUTOPHOSPHORYLATION OF CALCIUM/CALMODULIN-DEPENDENT PROTEIN KINASE II

Ca^{2+}/calmodulin-dependent protein kinase II is known to contain two subunits, α (M_r 50,000) and β (M_r 60,000/58,000), both of which undergo Ca^{2+}/calmodulin-dependent autophosphorylation. In the present study, we have studied the mechanism of this autophosphorylation reaction and its effect on the activity of the enzyme. Both subunits are autophosphorylated through an intramolecular mechanism. Using synapsin I as substrate, Ca^{2+}/calmodulin-dependent protein kinase II, in its unphosphorylated form, was totally dependent on Ca^{2+} and calmodulin for its activity. Preincubation of the enzyme with Ca^{2+}, calmodulin, and ATP, under conditions where autophosphorylation of both subunits occurred, converted the enzyme to one that was only partially dependent on Ca^{2+} and calmodulin for its activity. No change in the total activity, measured in the presence of Ca^{2+} and calmodulin, was observed. A non-hydrolyzable ATP analog did not substitute for ATP in the preincubation. Moreover, dephosphorylation of autophosphorylated Ca^{2+}/calmodulin-dependent protein kinase II with protein phosphatase 2A resulted in an enzyme that was again totally dependent on Ca^{2+} and calmodulin for its activity. It is proposed that autophosphorylation and dephosphorylation reversibly regulate the Ca^{2+} and calmodulin requirement of Ca^{2+}/calmodulin-dependent protein kinase II.

DISTRIBUTION OF PROTEIN III

Protein IIIa (M_r 74,000) and protein IIIb (M_r 55,000) are two major phosphoproteins found in mammalian brain. It was previously shown in intact nerve cells that the phosphorylation state of these two proteins could be increased by electrical stimulation, by depolarizing agents in the presence of calcium and by 8-bromo-cAMP. We now report that protein IIIa and protein IIIb possess significant structural homology as indicated by immunochemical studies using polyclonal and monoclonal antibodies and by peptide mapping studies. A quantitative radioimmunoassay using immunolabeling in SDS-polyacrylamide gels has been used to study the tissue distribution and regional and subcellular distribution in the brain of the two proteins. The two proteins were found only in nervous tissue and the adrenal medulla. Within the central nervous system, both proteins exhibited a distribution that parallels the relative density of nerve terminals. Subcellular fractionation studies indicated that both proteins are associated with synaptic vesicles.

DISTRIBUTION OF P38

An intrinsic protein of the membrane of brain synaptic vesicles with M_r-38,000 (p38) has recently been identified and partially characterized (Jahn, R., W. Schiebler, C. Ouimet and P. Greengard. 1985. Proc. Natl. Acad. Sci. U.S.A. 83, 4137-4141; Wiedenmann, B. and W. Franke, 1985, Cell 41, 1017-1028). In the present study we have used light microscopy immunocytochemistry and quantitative immunochemical techniques to systematically compare the distribution of p38 and synapsin I in nerve terminals of a variety of regions of the nervous system. We have also further characterized the distribution of p38 in nerve terminals by immunogold electron microscopy cytochemistry of hypothalamic synaptosomes. Our results suggest that p38, like synapsin I, is present in virtually all nerve terminals, and that in individual nerve terminals the two proteins are present in similar concentrations. Furthermore, p38, like synapsin I, is selectively associated with small synaptic vesicles (SSVs). No p38 was detectable on large dense-core vesicles (LDCVs), i.e., the secretory organelles involved in the storage and release of peptide neurotransmitters.

The findings indicate that at least two major components, one extrinsic (synapsin I) and one intrinsic (p38), of the membrane of SSVs are shared by SSVs of virtually all neurons, but not by LDCVs, support the idea that SSVs and LDCVs are organelles of two distinct pathways for regulated neuronal secretion.

MOLECULAR CLONING OF SYNAPSIN I

A partial clone for synapsin I was obtained, as previously reported. This clone was used as a probe to label a southern blot of rat genomic DNA digested independently with a number of restriction endonucleases. In each case, only a single band was labeled. This indicates that this clone is specific for only one sequence, and that this sequence represents a single-copy gene. The nucleotide sequence of this clone was determined and translated into a derived peptide sequence which closely matches the peptide sequence of the region of synapsin I flanking the site of phosphorylation by cAMP kinase and calcium/calmodulin kinase I. The minor discrepencies in sequence are believed to be due to species differences. The nucleotide sequence was derived from a rat cDNA clone, the peptide sequence was determined by direct peptide sequencing of the purified bovine protein. The rat clone was used to screen for full-length clones of synapsin I in a rat cDNA library with the Okayama-Berg vector, which enriches for full-length clones. A clone ~3000 bases in length was obtained. 1900 bases of this clone have been sequenced, and the putative peptide sequence has been derived. Several matches have been obtained between the sequence from the clone and the sequences of peptide fragments generated from purified snapsin I. This evidence indicates that this is a molecular clone for the cDNA of synapsin I.

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5. PROFESSIONAL PERSONNEL

Members of the Laboratory of Molecular and Cellular Neuroscience at the Rockefeller University who are associated with this research effort include:

Dr. Paul Greengard, Professor and Laboratory Head

Dr. Michael D. Browning, Assistant Professor

Dr. Reinhard Jahn, Assistant Professor

Dr. Angus C. Nairn, Assistant Professor

Dr. Charles C. Ouimet, Assistant Professor

Dr. Robert M. Lewis, Postdoctoral Associate

Dr. Teresa L. McGuinness, Guest Investigator

Ms. Yvonne Lai, Guest Investigator

6. INTERACTIONS

Papers presented at scientific meetings:

Greengard, P. (1985) Protein phosphorylation in the brain. Plenary Lecture. Abstracts of Ninth European Neurosci. Congress, Oxford, Neurosci. Lett. S205.

Navone, F., Jahn, R., Schiebler, W., Greengard, P. and DeCamilli, P. (1985) Comparison of the distributions of synapsin I and of a major intrinsic membrane protein (P38) of synaptic vesicles in brain and other tissues. Abstracts of Ninth European Neurosci. Congress, Oxford, Neurosci. Lett. S231.

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Navone, F., Jahn, R., Di Gioia, G., Greengard, P. and De Camilli, P. (1986) The synaptic vesicle membrane protein P38 is present also in endocrine cells, and in both neurons and endocrine cells is selectively associated with vesicles distinct from peptide containing granules. Abstract for Second European Congress on Cell Biology.

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